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Bark-Inhabiting Green Algae in Japan (1) *Scenedesmus komarekii* and *Coelastrella multistriata* var. *multistriata* (Scotiellocystoideae, Chlorellaceae, Chlorophyceae)

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Three bark-inhabiting green algae were collected from the Uchibo area, Chiba Prefecture, Japan. They form secondary carotenoids and are morphologically similar to the genus *Chlorella*. Two of the isolates have spines on cell surface and form 4-celled coenobium in a medium by addition of glucose (0.5%). They were identified with *Scenedesmus komarekii* Hegewald based on the ornamentation pattern of the cell surface. The third alga has meridional ribs on cell surface, a characteristic of the genus *Coelastrella*. It was identified with *C. multistriata* (Trenkwalder) Kalina & Punčochářová var. *multistriata* mainly on the basis of the shape of autospores. These two species are newly reported from Japan.

Introduction

The algal flora on barks of trees is subjected to environmental conditions that are more rapidly changing than in aquatic habitats. Quite a few phycologists have studied corticolous algae. Most of the studies were floristic or ecological, and a few are taxonomic at species level. Our knowledge concerning the species composition of epiphloeodic algal flora is, therefore, far from sufficient.

We collected many specimens of bark inhabiting microalgae in Uchibo area, Chiba Prefecture. They were cultured and examined with light and electron microscopy. Of these, three strains of green algae roused our interest since they resembled *Chlorella*, but formed secondary carotenoids. It prompted studies of not only their morphology and cellular structure, but also the composition of photosynthetic pigments. After examination, two of them were identified as *Scenedesmus komarekii* Hegewald (Hegewald 1982), and the other was identified as *Coelastrella multistriata* (Trenkwalder) Kalina & Punčochářová var. *multistriata* (Kalina and Punčochářová 1987).

Materials and Methods

The three strains were tentatively designated as strains AW-2, A3-2 and C6-2. Strains AW-2 and A3-2 were collected on barks of *Zelkova serrata* (Thunb.) Makino at Anegasaki, Ichihara City, on 10 October, 1992, and Inage, Chiba City, on 5 June, 1993, respectively. Strain C6-2 was collected on a bark of *Cryptomeria japonica* D. Don., in Nagae, Chosei Gun, on 23 September, 1993. The strains were cultured in modified Fitzgerald medium as shown in Table 1, and after the unialgal culture was established, they were maintained at 25°C on agar slants made up using Bold's basal medium (BBM) (Bold 1949).

Algal strains used for microscopy and pigment analysis were cultured in BBM at 25°C on a rotary shaker (100 rpm). The description is based on the materials in the culture with BBM at 25°C unless mentioned otherwise. The cultures were illuminated at 100 μ Einsteins $m^{-2}s^{-1}$. For comparison, *Graesiella vacuolata* UTEX 251 and *Scenedesmus fuscus* UTEX 343 provided from the Culture Collection of Algae, at the University of Texas at Austin, USA, and *Scenedesmus komarekii* strain Hegewald 1977-165 which was collected from Lake Langui in Peru provided from Dr. Eberhard Hegewald at Institute of Biotechnology, Jülich in Germany, were cultured in the same medium and under the same conditions.

For scanning electron microscopy, algal strains were fixed with 2% glutalaldehyde and 1% OsO_4. The algae were washed with distilled water for 2 hours, and then centrifuged at 3000 rpm for 10 min. The algae harvested were dehydrated using elevated ethanol solution from 20 to 100% for each 20 min. The pellets were treated with 50 and 100% isoamyl acetate, and then dried to critical point. The samples were coated with Pt (approx. 20 nm) in ion spattering. Scanning electron microscopic observation was carried out using JSM-6400F (JEOL, Tokyo). The method for transmission electron microscopy was that of Chihara et al. (1994), except that NaCl was not included in the buffer.

Table 1. Chemical composition of modified Fitzgerald medium

$NaNO_3$	0.496 g
K_2HPO_4	0.039 g
$MgSO_4\,7H_2O$	0.075 g
$CaCl_2\,2H_2O$	0.036 g
Fe-citrate	0.006 g
EDTA-Na	0.001 g
Gaffron solution[a)]	1 ml
Distilled water	1000 ml

[a)] Gaffron solution: H_3BO_3 3.1 g, $MnSO_4\,4H_2O$ 2.23 g, $ZnSO_4\,7H_2O$ 0.287 g, $(NH_4)6Mo_7O_{24}\,4H_2O$ 0.088 g, KBr 0.199 g, Kl 0.083 g, $Cd(NO_3)_2SO_4\,6H_2O$ 0.198 g, $VOSO_4\,2H_2O$ 0.02 g and $Al_2(SO_4)_3\,K_2SO_4\,24H_2O$ 0.474 g in 1000 ml of N/10 H_2SO_4.

These strains examined are maintained at Chiba Laboratory, Mitsui Engineering & Shipbuilding Co. Ltd.

Pigment composition was analyzed using the reverse-phase HPLC. The HPLC system (Tosoh, Tokyo) consisted of a CCMP pump, a system controller SC-8010, and an UV detector UV-8010. Cells were collected by centrifugation and washed with distilled water twice. The pigments were extracted with cold acetone/methanol (7:3, v/v) for 1 h after grinding the algal cells with a pestle. The extract was centrifuged, and 100 μl of supernatant was injected into a TSK gel ODS-80TM column (250 mm × 4.6 mm, Tosoh). The sample was eluted with methanol at flow rate of 1 ml min^{-1}. The pigments were detected by the absorbance at 450 nm. The retention times of each peak were compared with those from reference of Skoda (1992) and authentic canthaxanthin and astaxanthin.

Results

Morphology and cellular structure

Scenedesmus komarekii Hegewald Strain AW-2

(Figs. 1, 2)

Light microscopy

The alga is solitary, unicellular, spherical, 4–14 μm in diameter, or ovoid to ellipsoidal, 4–12×3–8 μm in size. Young cells are sometimes tetrahedric. Cells are yellow-green in logarithmic phase of growth and brick red in old culture, and larger in size at 40°C than at 25°C. Each cell has a single parietal and saucer-, cup- or girdle-shaped chloroplast with a pyrenoid near the base. The pyrenoid is spherical to ovoid and surrounded by bipartite starch grains. Oil-like droplets are numerous in the cytoplasm and red granules are sometimes present. Vacuoles are sometimes

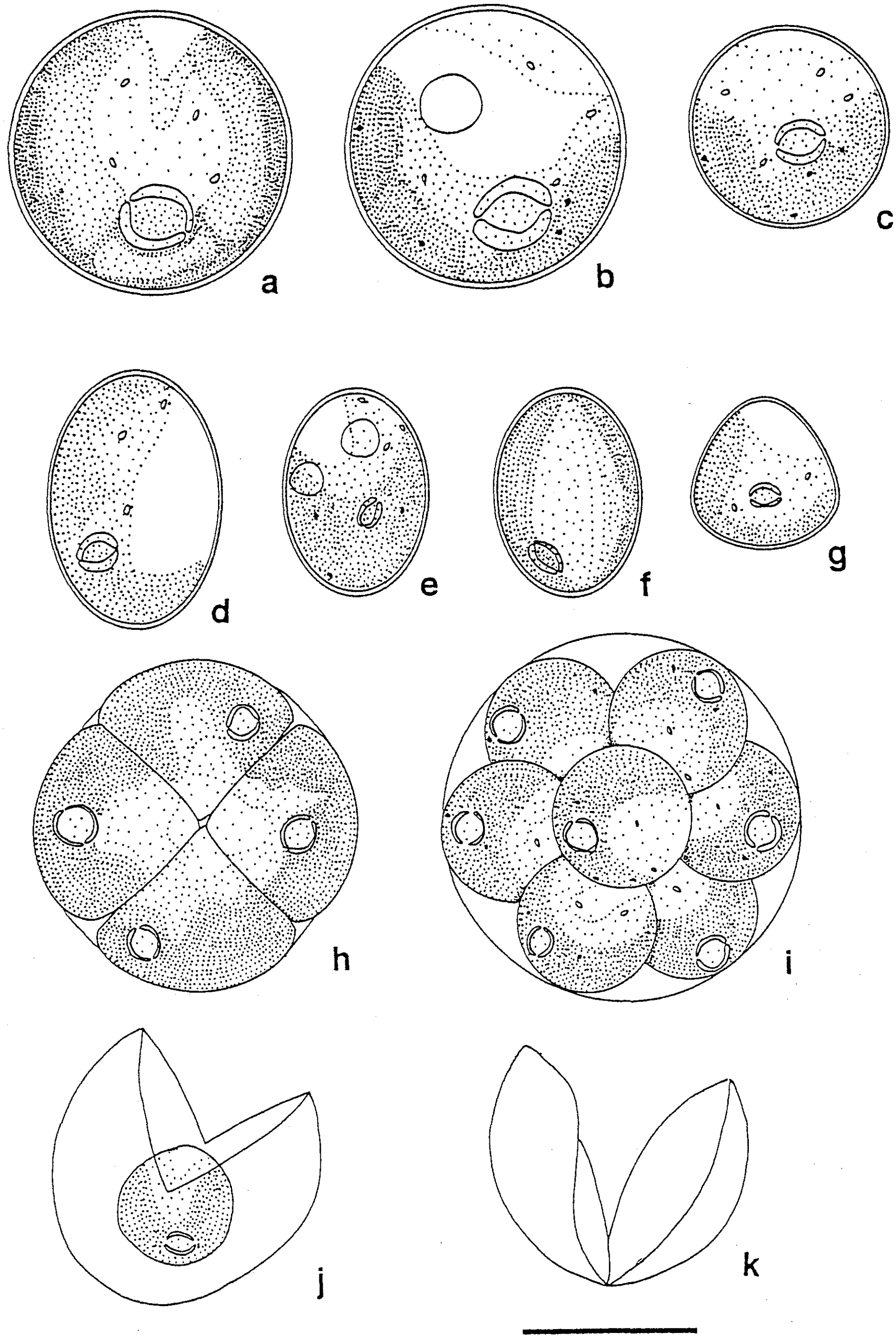

Fig. 1. *Scenedesmus komarekii*, strain AW-2. a. Cell with a girdle-shaped chloroplast. b, c. Cell with a cup-shaped chloroplast. d–f. Young ellipsoidal cells. g. Young roundly tetrahedric cell. h, i. Autospores in sporangium. j. Empty mother cell wall with the last autospore inside. k. Empty mother cell wall disintegrated into two parts. Scale bar=5 μm.

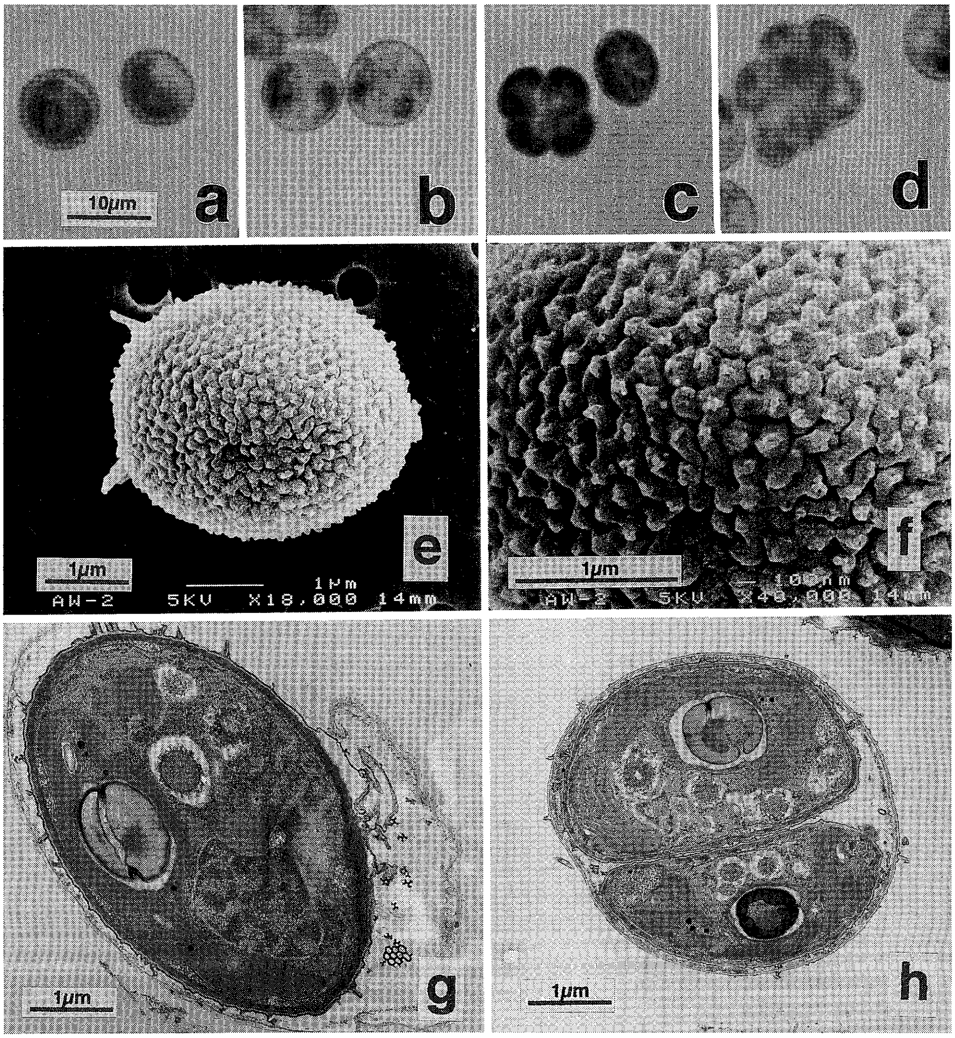

Fig. 2. *Scenedesmus komarekii*, strain AW-2. (a–d. Photomicrographs, e, f. scanning electron micrographs, g, h. transmission electron micrographs). a. Vegetative cells. b. Cells with cleavage of the protoplast into two and four parts. c. Sporangium with four autospores. d. Autospores discharged from mother cell. e. Vegetative cell with numerous warts and two spines on the cell surface. f. Warts. g. Section of vegetative cell showing distribution of major cell organelles. h. Section of sporangium showing formation of autospores.

present, but are not observed when the alga is cultured on agar medium.

Cells are capable of producing 2–16 autospores. Reproduction begins with cleavage of the protoplast into two parts of almost equal portions in size, followed by the second cleavage which takes place perpendicularly to the first formed cleavage. Each of the four cells thus formed undergoes a similar division, resulting in the formation of 8–16 autospores. Autospores are spherical, 3–7 μm in diameter, ovoid or roundly tetrahedric, 3–7 × 3–6 μm in size. The mother cell wall forms a split through which the autospores are discharged, and then mother cell wall is almost completely disintegrated into two parts. Autospores can remain within the empty mother cell.

Electron microscopy

Cells have numerous warts and 2–4 short spines on the surface of cell wall. Each wart consists of 3–5 short tubes. The spine is 0.3–0.5 μm in height and is composed of 3–10 tubes of various length.

Scenedesmus komarekii Hegewald Strain A3-2 (Figs. 3, 4)

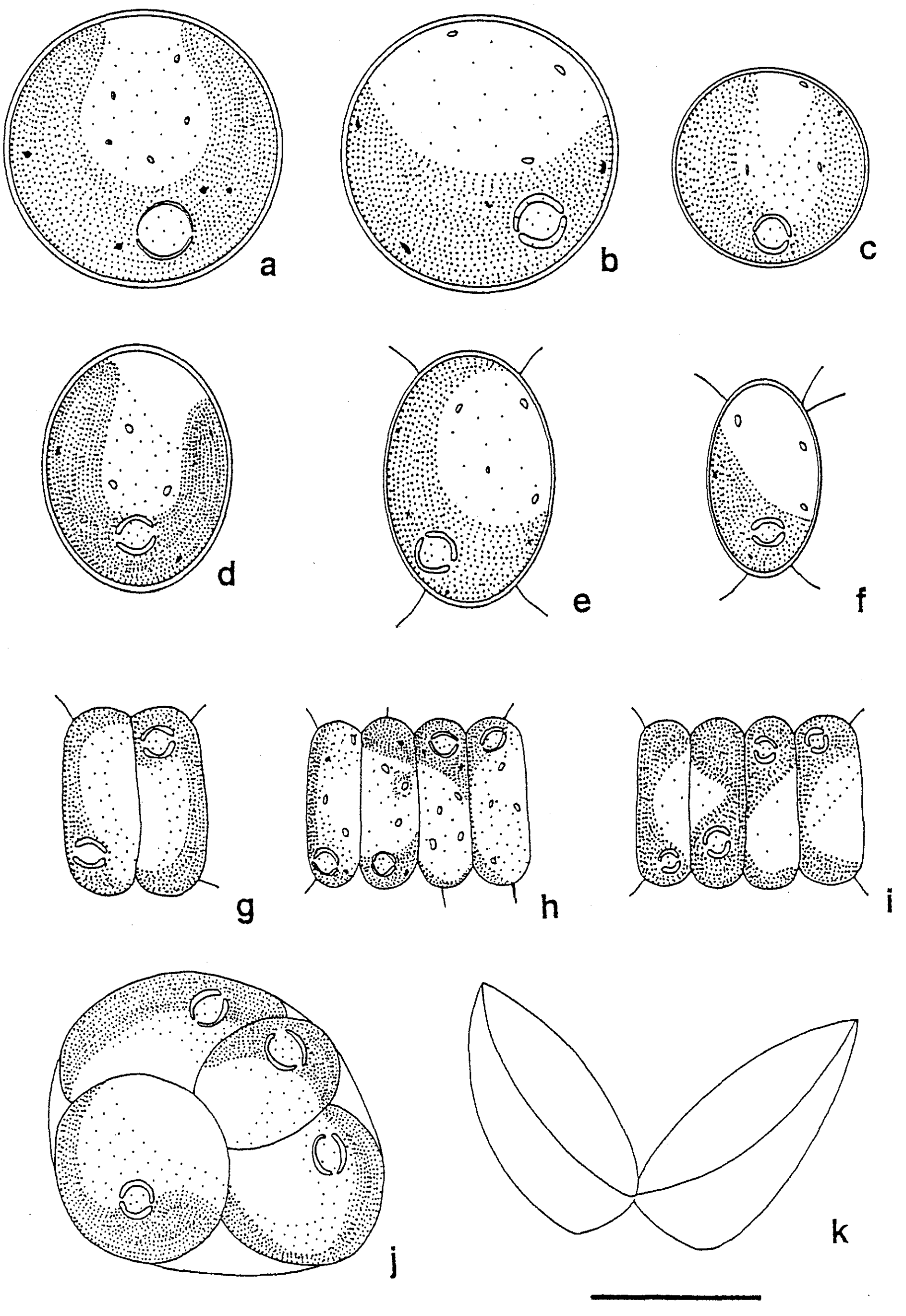

Fig. 3. *Scenedesmus komarekii*, strain A3-2. a, b. Cells with a cup-shaped chloroplast. c. Cell with a girdle-shaped chloroplast. d. Ellipsoidal cell with a cup-shaped chloroplast. e, f. Ellipsoidal cells with spines. g. Two-celled coenobium with spines. h, i. Four-celled coenobia with spines. j. Autospores in sporangium. k. Empty mother cell wall disintegrated into two parts. Scale bar=5 μm.

Light microscopy

The alga is usually unicellular although rarely 2-celled or 4-celled coenobia are formed. Cells in the unicellular phase are spherical, 5–12 μm in diameter, or ovoid 4–8×3–5 μm in size, and sometimes possess 4–6 spines while those in coenobium are ellipsoidal, 3–6×2–4 μm in size and linearly arranged, and possess 2–4 spines. Cells in both phases are yellow-green in logarithmic phase of growth and brick red in old culture. Each cell has a single parietal and saucer-, cup- or girdle-shaped chloroplast with a pyrenoid near the base. The pyrenoid is spherical or ovoid, and surrounded by bipartite starch grains. Oil like droplets are numerous and vacuoles are not observed.

In liquid culture at 40°C, alga is always unicellular, and ovoid to ellipsoidal, 4–12×3–8 μm in size.

On agar medium, the alga is mostly unicellular. Cells are ovoid to ellipsoidal, 4–9×3–5 μm in size, and

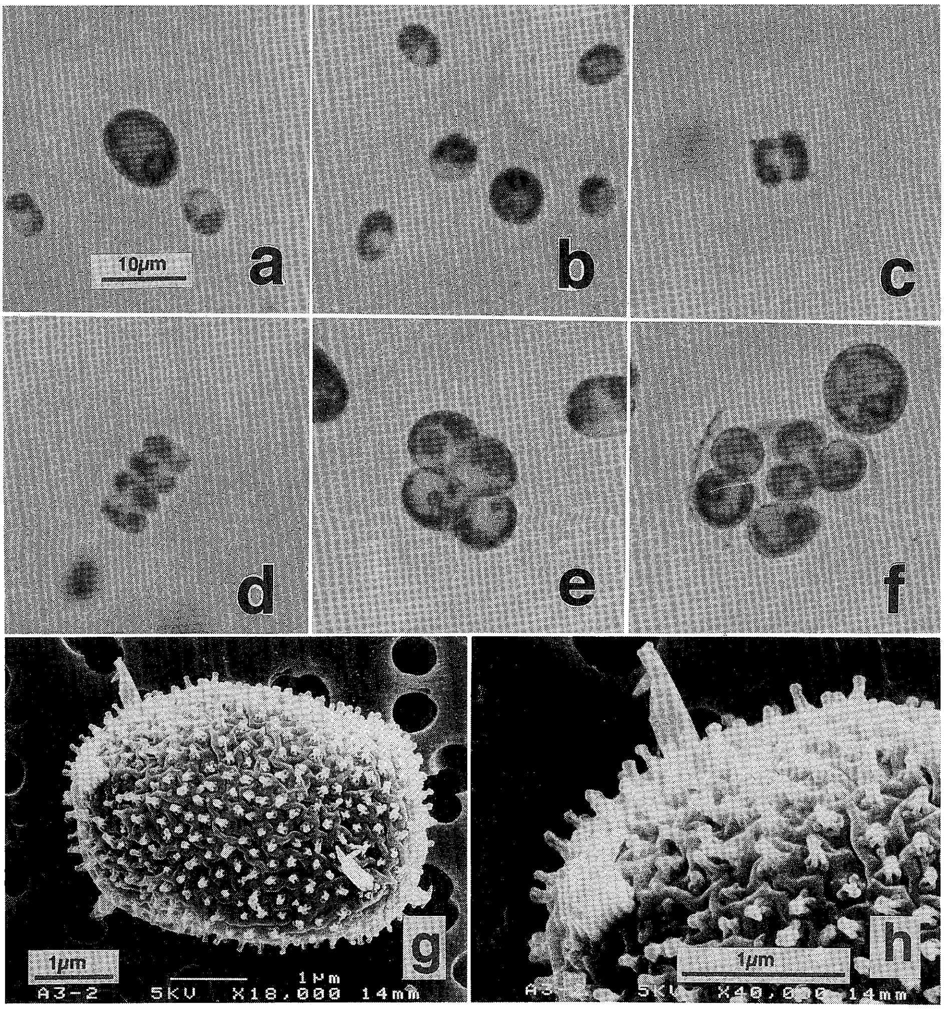

Fig. 4. *Scenedesmus komarekii*, strain A3-2. (a–f. Photomicrographs, g, h. scanning electron micrographs). a. Vegetative cells. b. Vegetative cell with spines. c. Two-celled coenobium. d. Four-celled coenobium. e. Sporangium with four autospores. f. Autospores discharged from mother cell. g. Vegetative cell with numerous long warts and four spines on the cell surface. h. Part of the cell surface showing spines, long warts and shoe brush-shaped appendage.

have 3–7 spines on the surface of cell wall. Spines are 1.5–3 μm in length. Spined cells are rarely observed in old culture.

Reproduction takes place by successive cell divisions and 2–16 autospores are formed. Autospores are spherical, 3–5 μm in diameter, ovoid or roundly tetrahedric, 4–7×3–4 μm in size. The mother cell wall forms a split through which autospores are discharged, and then mother cell wall nearly disintegrates into 2–4 parts. Autospore can remain within the empty mother cell.

Electron microscopy

Cells have numerous long warts and 3–7 spines all over the cell surface. The long wart is composed of 4–5 long tubes which are 0.15–0.25 μm in height. The spine is about 1.0 μm in height and composed of 5–10 tubes of various length. Cells have shoe brush-shaped appendages rarely on the cell surface. The appendage is 0.3–0.5 μm in height and consists of more than 10 tubes.

Coelastrella multistriata **(Trenkwalder) Kalina & Punčochářová var.** ***multistriata*** **Strain C6-2** (Figs. 5, 6)

Light microscopy

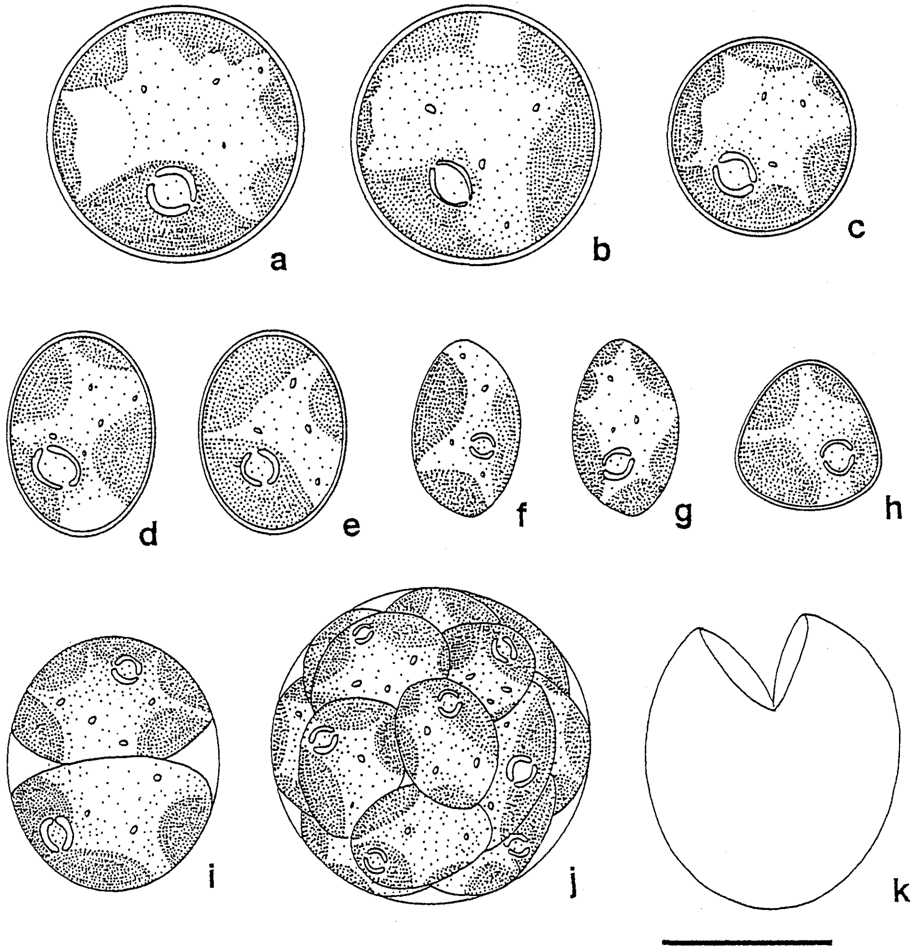

Fig. 5. *Coerastrella multistriata* var. *multistriata*, strain C6-2. a–c. Cells with separated chloroplasts. d, e. Young ellipsoidal cells. f, g. Young citriform cells. h. Young roundly tetrahedric cell. i, j. Autospores in sporangium. k. Empty mother cell wall. Scale bar=5 μm.

The alga is unicellular, solitary or in aggregate. Young cells are usually ovoid or in citriform, 6–10×3–7 μm in size, and mature cells are spherical, 6–10 μm in diameter, and are yellow-green in logarithmic phase of growth while brick red in old culture. They have a single chloroplast that is parietal and separated into many parts, with a pyrenoid. The pyrenoid is spherical to ovoid, and surrounded by a biplate starch grains. Oil-like droplets are numerous and vacuoles are not observed.

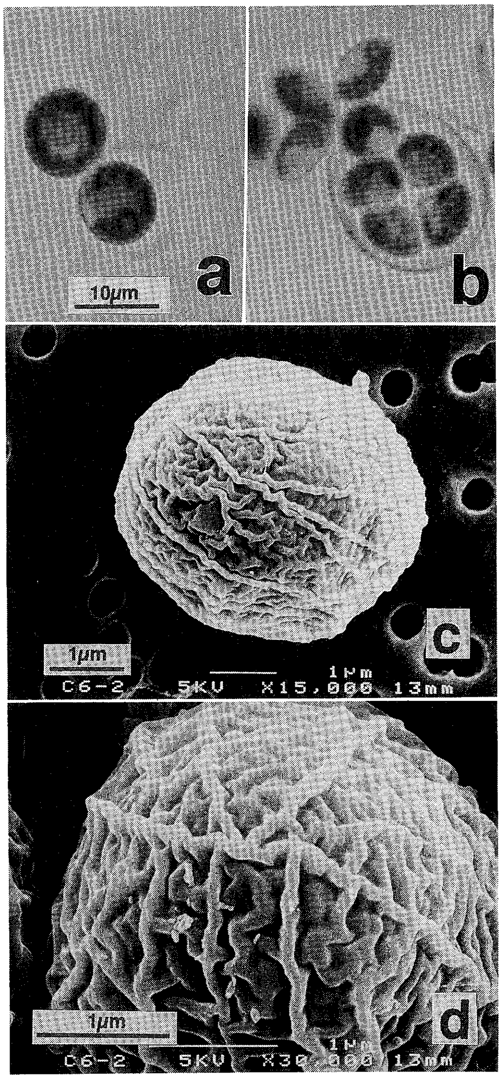

Fig. 6. *Coerastrella multistriata* var. *multistriata*, strain C6-2. (a, b. Photomicrographs, c, d. scanning electron micrographs). a. Vegetative cells. b. Autospores discharged from mother cell. c. Vegetative cell with meridional ribs on the cell surface. d. Polar region of the cell.

Reproduction is by 2–16 autospores within a cell. Autospores are spherical, 3–5 μm in diameter, ovoid or citriform, 5–11×3–5 μm in size. The mother cell forms an aperture through which the autospores are liberated.

Electron microscopy

The alga has 10–25 meridional ribs on the outer cell wall. Ribs are relatively smooth and without polar thickenings.

Photosynthetic pigments

The composition of photosynthetic pigments is fundamentally identical in the three strains (Fig. 7). In logarithmic phase of growth, chlorophylls a and b, β-carotene, neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and echinenone were detected. In old culture, astaxanthin, canthaxanthin and other unidentified pigments were detected in addition to the primary pigments in all the three strains.

Discussion

Strain AW-2 is close to *Chlorella fusca* Shihira & Kraus var. *fusca* and var. *vacuolata* Shihira & Kraus in gross morphology including the shape and size of cells, the method of autospore formation and cell pigmentation. The type variety has been transferred into the genus *Scenedesmus* because of the similarity of cell wall structure; e.g., the presence of spines and characteristic ornamentation on the surface of cell wall (Hegewald 1982). On the other hand, var. *vacuolata* is now a member of *Graesiella*, the genus established by Kalina and Punčochářová (1987) on the basis of the trilaminar structure of cell wall, the presence of a network of fine ribs on the cell surface and the formation of secondary carotenoids.

As described above, strain AW-2 has spines on the cell surface, a characteristic feature of *Scenedesmus*. One of the primarly characters for circumscription of

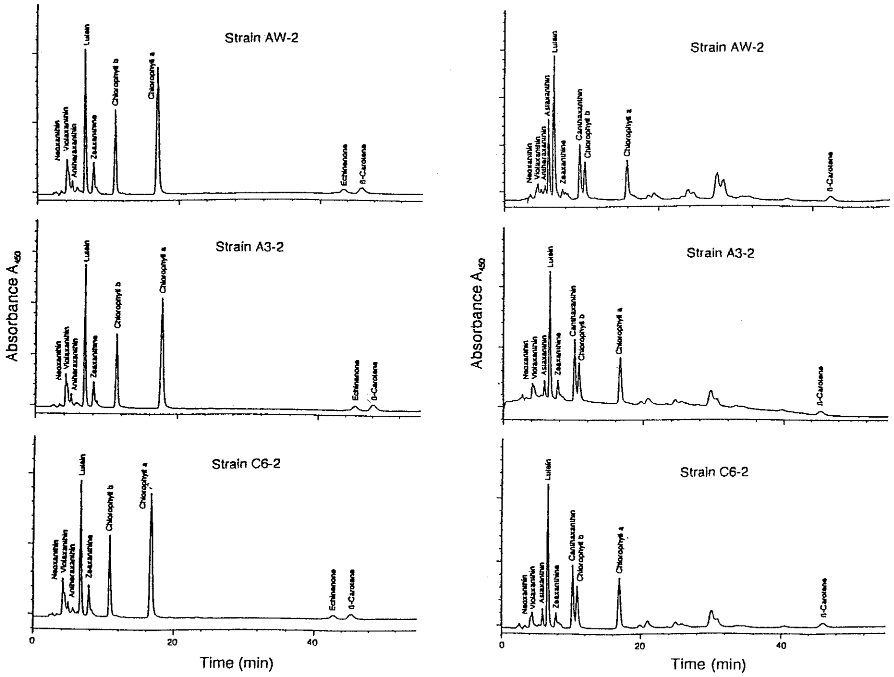

Fig. 7. HPLC chromatograms of photosynthetic pigments extracted from the cells of three strains of logarithmic phase of growth (left) and in old cultures (right). AW-2, *Scenedesmus komarekii*; A3-2, *Sc. komarekii*; C6-2, *Coerastrella multistriata* var. *multistriata*.

the genus is the 4-celled coenobium. According to Hegewald (personal communication), single cells of *Scenedesmus* tend to become coenobia in a medium containing organic carbon. Coenobium formation of strain AW-2 was induced in this study by addition of glucose (0.5%) into BBM (Fig. 8).

The ornamentation of cell surface is the most important character for identification at species rank of *Scenedesmus*. The cell wall ornament of strain AW-2 revealed by electron microscopy resembles that of *Scenedesmus komarekii* Hegewald reported by Komárek (1987) and Hegewald (1989). For better understanding of strain AW-2, *Sc. komarekii* strain Hegewald 1977-165 from Hegewald was examined by the scanning electron microscope (Fig. 9). Warts and spines in strain AW-2 was fundamentally identical in shape with those of strain Hegewald 1977-165 although warts were somewhat shorter in strain AW-2.

At first sight, strain A3-2 was thought of a species of the genus *Chlorella*, because the alga was almost unicellular. The strain, however, occasionally formed 2-celled and 4-celled coenobia and the single cells sometimes had long spines. These features corresponded to those of *Scenedesmus*. The cell surface observed by the scanning electron microscope showed that warts and spines were basically the same both in shape and in size as those of strain Hegewald 1977-165.

According to Hegewald (1989), there are two patterns of cell surface ornaments in *Sc. komarekii*. The one, which is more commonly observed, has long warts while the other has short warts called "armed warts" in which long warts are reduced. The strains of A3-2 and Hegewald 1977-165 have the common type of warts while strain AW-2 has reduced type of warts. It should be noticed that strain AW-2 has the reduced warts and no potential of forming coenobia in au-

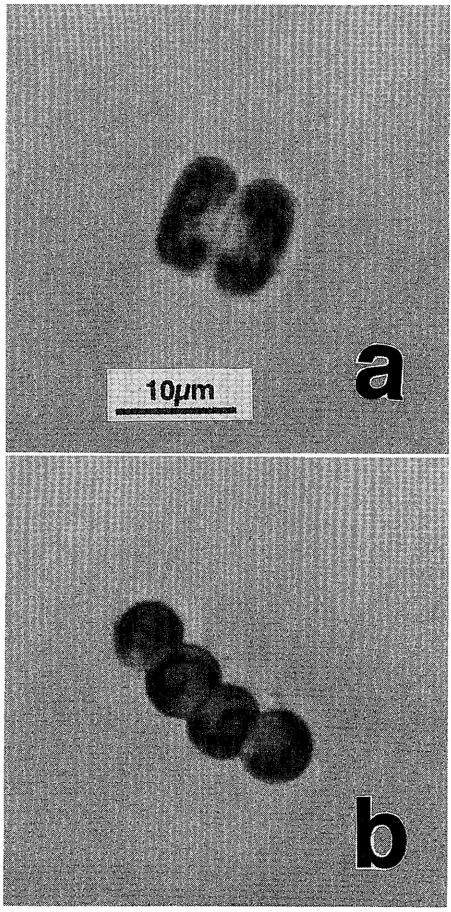

Fig. 8. Coenobia of *Scenedesmus komarekii* strain AW-2 formed in BBM medium added with 0.5% glucose.

totrophic condition. We, however, consider at present those to be a strain difference and we treat two strains studied as *Sc. komarekii.*

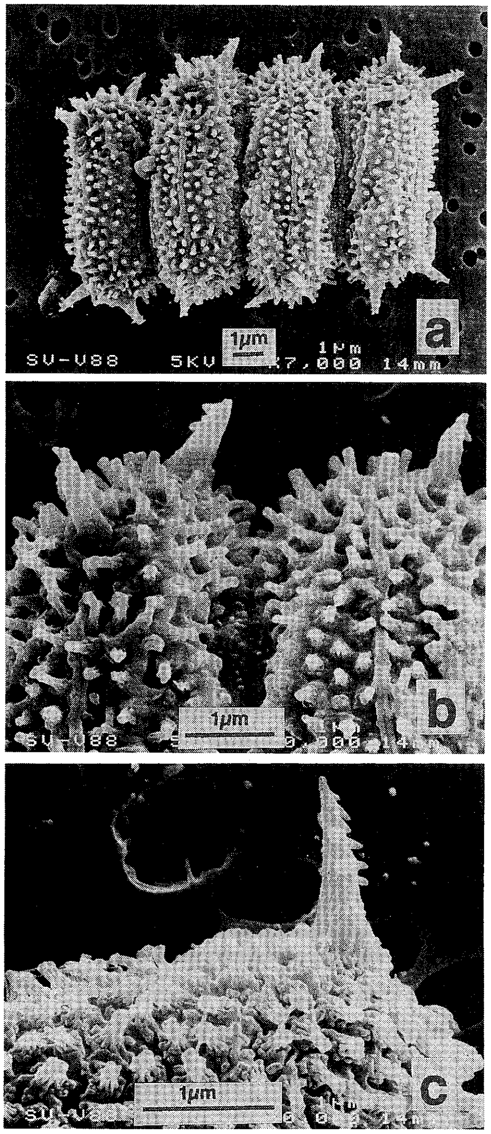

Fig. 9. Scanning electron micrographs. *Scenedesmus komarekii* strain Hegewald 1977-165. a. Vegetative four-celled coenobium. b. Long warts and spines. c. Spines.

Strain C6-2 was thought to be a species of *Chlorella* since the alga was unicellular without spines on the cell surface. We later recognized it to be a member of subfamily Scotiellocystoideae of Chlorellaceae, because the alga formed secondary carotenoids. Observation with the scanning electron microscope revealed that the strain had numerous meridional ribs covering the entire surface of cell without thickenings at the polar regions. This feature is one of the primary characters for the genus *Coelastrella.* Kalina and Punčochářová (1987), who recognized four species and one variety; *C. striolata* Chodat, *C. compacta* Skuja, *C. levicostata* Korsikov, *C. multistriata* (Trenkwalder) Kalina & Punčochářová var. *multistriata* and var. *corcontica*, and provided a key to these taxa of *Coelastrella.* They used the following features as main criteria; the presence or absence of the colonial form, the shape of autospores, and the presence or absence of dentation on the ribs. Strain C6-2 is always solitary and produces ovoid and citriform autospores. According to Kalina and Punčochářová, an alga with these two features falls into *C. multistriata* var. *multistriata.*

Scenedesmus komarekii was first isolated from a desicated soil samples in Connecticut, USA, and strain Hegewald 1977-165 was isolated from a lake. According to Hegewald (personal communication), this species is apparently rare in lakes and common in terrestrial habitats. The type strain of *C. multistriata* var. *multistriata* was isolated from soil of pine wood, Brixen region, Southern Tyrol, Italy. This is the first report to these two algae as inhabitants on tree barks. This is also the first record of these two algae in Japan.

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References

Bold H. 1949. The morphological of *Chlamydomonas chlamydogama* sp. nov. Bull. Torrey Bot. Club **70**: 101–108.

Chihara M., Nakayama T., Inouye I. and Kodama M. 1994. *Chlorococcum littorale*, a new marine green coccoid alga (Chlorococcales, Chlorophyceae). Arch. Protistenkd. **144**: 227–235.

Hegewald E. 1982. Taxonomic-morphological studies of *Scenedesmus* isolates from culture collections. Algological Studies **29**: 375–406.

——— 1989. The *Scenedesmus* strains of the culture collection of the University of Texas at Austin (UTEX). Algological Studies **55**: 153–189.

Kalina T. and Punčochářová M. 1987. Taxonomy of the subfamily Scotiellocystoideae Fott 1976 (Chlorellacaceae, Chlorophyceae). Algological Studies **45**: 473–521.

Komárek J. 1987. Species concept in coccal green algae. Algological Studies **45**: 437–471.

Skoda B. 1992. Contribution to the biochemical taxonomy of the genus *Chlorella* Beijerinck sensu lato – pigment composition. I. Cultures growing under optimal conditions. Algological Studies **63**: 19–35.

花方信孝，軽部征夫，千原光雄：**日本産樹皮付着性緑藻（1）**

内房地区（千葉県）の木樹皮より単離した緑藻類の中で，形態が **Chlorella** 属に類似するが二次カロチノイドを生成する3株について分類学的研究を行った．電子顕微鏡観察により2株は ***Scenedesmus*** 属に特徴的な刺状突起を細胞表面に持つことがわかり，さらにこれらは細胞表面に特徴的な模様をもつことから ***Sc. komarekii*** **Hegewald** と同定した．残る1株は細胞表面に子午線状の肋骨状の突起を有することから ***Coelastrella*** 属であり，さらに自生胞子の形態的特徴から ***C. multistriata*** **（Trenkwalder） Kalina & Punčochářová var.** ***multistriata*** と同定した．これらは樹皮付着性緑藻として初めて知られ，またいずれも日本新産であった．